**Minor modifications were made to this method to accommodate existing laboratory equipment and related procedural requirements.**

**Key to Revisions:**

**Deletions - Strike-through**
**Additions - Bold and underlined**

# RM 35 - Determination of total alkaloids in tobacco by continuous flow analysis

## CORESTA RECOMMENDED METHOD N° 35

## DETERMINATION OF TOTAL ALKALOIDS (AS NICOTINE) IN TOBACCO BY CONTINUOUS FLOW ANALYSIS
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### 0. Introduction

Studies carried out by a CORESTA Task Force between 1989 and 1993 have shown that the two procedures for determination of total alkaloids in tobacco as described in CORESTA Recommended Method N° 20 and the present Method may not produce identical results for some dark tobaccos or those containing significant levels of alkaloids other than nicotine.

The studies have indicated that these differences may be due to the fact that the recoveries and detection sensitivities of the two methods towards the alkaloids other than nicotine are different.

Therefore, when reporting results it is important to specify the method used.

### 1. Field of Application

This method is applicable to unmanufactured and manufactured tobacco.





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## 2. References

CORESTA Recommended Method N° 20:1968
*Determination of alkaloids in manufactured tobacco.*

CORESTA Recommended Method N° 39:1994
*Determination of the purity of nicotine and nicotine salts by gravimetric analysis - Tungstosilicic acid method.*

## 3. Principle

An aqueous (see note 1) extract of the tobacco is prepared and the total alkaloids (as nicotine) content of the extract is determined by reaction with sulphanilic acid and cyanogen chloride. Cyanogen chloride is generated in situ by the reaction of potassium cyanide and chloramine T (see appendix 1). The developed colour is measured at 460 nm.

**Note 1 :** Collaborative studies have shown that this method gives equivalent results for water and 5% acetic acid extracts. It is recommended that 5% acetic acid extracts should be used if total alkaloids (as nicotine) and reducing substances (see CORESTA Recommended Method N° 37) or reducing carbohydrates (see CORESTA Recommended Method N° 38) analysis are to be carried out simultaneously.

## 4. Safety Precautions

Potassium cyanide is poisonous and irritant, thus all safety precautions must be observed when handling this material. Solutions shall be prepared by a designated responsible person. Gloves and safety glasses shall always be used when making up solutions and bottles of the made-up reagent shall always be carried in a suitable safety carrier. ~~To prevent the escape of vapour into the laboratory, reagent pick-up tubes shall pass through a soda-lime trap into the reagent bottle (see diagram 2).~~

~~The cyanide neutralising agents A and B are pumped as shown in the flow diagram (see diagram 1) and mixed in a 2 $dm^3$ Buchner flask with magnetic stirring (see diagram 3). All waste solutions containing cyanogen chloride are run into this flask where conversion to the "Prussian Blue" complex occurs. The contents of the Buchner flask are allowed to over-flow into a storage flask, the contents of which are stored overnight in a fume cupboard and then disposed of as waste.~~

**Run all lines from the alkaloid colorimeter and debubbler into a waste vessel containing a caustic hypochlorite solution to oxidize the cyanide to cyanate. The waste should be handled for disposal by trained personnel.**



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~~Suitable cyanide poisoning treatment kits are available from laboratory suppliers and shall be located in the vicinity of the analyzer to be used by a competent person.~~

## 5. Reagents

All reagents shall be used according to good laboratory practice and existing national regulations.

**5.1** *Brij 35 Solution (Polyoxyethylene Lauryl Ether)*

Add 1 $dm^3$ distilled water to 250 g Brij 35, warm and stir until dissolved.

**5.2** *Buffer Solution A*

Dissolve 2.35 g sodium chloride (NaCl) and 7.60 g sodium tetraborate ($Na_2B_4O_3.10H_2O$) in distilled water. Transfer to a 1 $dm^3$ volumetric flask, add 1 $cm^3$ Brij 35 solution (5.1) and dilute to volume with distilled water. Filter the solution through a Whatman N° 1 (or equivalent) filter paper before use.

**5.3** *Buffer Solution B*

Dissolve 26 g anhydrous disodium hydrogen orthophosphate ($Na_2HPO_4$), 10.4 g citric acid ($COH(COOH)(CH_2COOH)_2.H_2O$) and 7 g sulphanilic acid ($NH_2C_6H_4SO_3H)_2$ in distilled water, transfer to a 1 $dm^3$ volumetric flask, add 1 $cm^3$ Brij 35 solution (5.1) and dilute to volume with distilled water. Filter the solution through a Whatman N° 1 (or equivalent) filter paper before use.

**5.4** *Chloramine T Solution (N-chloro-4-methyl benzenesulphonamide sodium salt), ($CH_3C_6H_4SO_2N(Na)Cl.3H_2O$)*

Dissolve 8.65 g chloramine T in distilled water, transfer to a 500 $cm^3$ volumetric flask and dilute to volume with distilled water. Filter the solution through a Whatman N° 1 (or equivalent) filter paper before use.

~~**5.5** *Cyanide Neutralising Solution A*~~

~~Dissolve 1 g citric acid (5.3) and 10 g ferrous sulphate ($FeSO_4.7H_2O$) in distilled water and dilute to 1 $dm^3$.~~

~~**5.6** *Cyanide Neutralising Solution B*~~

~~Dissolve 10 g anhydrous sodium carbonate ($Na_2CO_3$) in distilled water and dilute to 1 $dm^3$.~~



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**Caustic Hypochlorite Solution**

**Dissolve approximately 1 g sodium hydroxide in 300 mL of water in a 10-liter plastic bottle. Add approximately 100 mL of Clorox™.**

5.7 *Potassium Cyanide Solution (KCN)*

**CARE: POTASSIUM CYANIDE IS EXTREMELY TOXIC! SEE SAFETY PRECAUTIONS.**

In a fume cupboard, weigh 2 g potassium cyanide into a 1 $dm^3$ beaker. Add 500 $cm^3$ distilled water and stir (magnetic stirrer) until all of the solid has dissolved. Store in a brown glass bottle.

~~5.8 *Nicotine Hydrogen Tartrate ($C_{10}H_{14}N_2(C_4H_6O_6)_2 \cdot 2H_2O$) for the Preparation of Standards*~~
***l*-Nicotine; $C_{10}H_{14}N_2$; F.W. 162.23. 99+% purity (e.g. Acros 18142-0250).**

5.9 *Standard Nicotine Solutions*

~~5.9.1 Check the purity of the nicotine hydrogen tartrate according to CORESTA Recommended Method N° 39.~~

~~5.9.2 Stock Solution: Weigh, to the nearest 0.0001 g, approximately 1.3 g of nicotine hydrogen tartrate in distilled water and dilute to 250 $cm^3$ in a volumetric flask. This solution contains approximately 1.6 mg nicotine per $cm^3$. Store in a refrigerator. Prepare a fresh solution every month.~~

**Stock Solution, 1.5 mg/mL: Weigh 0.750 g of nicotine and transfer into a 500 mL volumetric flask. Dissolve and dilute to volume with the 5% acetic acid extraction solution. After opening, date and store the unused portion in a plastic container in the refrigerator.**

~~5.9.3 Working Standards: From the stock solution produce a series of at least five calibration solutions whose concentrations cover the range expected to be found in the samples *e.g.* 0.04-0.80 mg nicotine per $cm^3$. Calculate the exact concentration for each standard taking into account the purity of the nicotine hydrogen tartrate. Store in a refrigerator. Prepare fresh solutions every two weeks.~~

**Working Standards: Pipet into six separate 100 mL volumetric flasks 1, 3, 5, 7, 10, and 15 mL of the nicotine standard stock. Dilute all to volume with the 5% acetic acid extraction solution. Each flask contains 0.015, 0.045, 0.075, 0.105, 0.150, and 0.225 mg/mL of nicotine respectively.**



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**Note 2 :** The method can also be standardized by using nicotine or other nicotine salts of known purity. In this case an amount equivalent to the above used nicotine hydrogen tartrate shall be used.

## 6. Apparatus

**6.1** The necessary general laboratory equipment, for the preparation of samples, standards and reagents.

**6.2** Continuous flow analyzer (see diagram 1) consisting of:

Sampler
Proportioning pump
Dialyser
Delay coils
Colorimeter (or equivalent) with 460 nm filter(s)
Recorder

**6.3** Coil for cyanogen chloride generation

A commercially available microbore mixing coil can be used for the *in situ* generation of cyanogen chloride. Alternatively a ~~five-turn~~ mixing coil can be prepared (see appendix 2)

## 7. Analysis of Tobacco Samples

**7.1** Prepare the tobacco for analysis by grinding (the sample should totally pass through a 1 mm sieve) and determine the moisture content. If the tobacco is too wet for grinding it can be dried at a temperature not exceeding 40 °C.

~~**7.2** Weigh, to the nearest 0.0001 g, approximately 250 mg of the tobacco in a 50 $cm^3$ dry conical flask. Add 25 $cm^3$ distilled water, stopper the flask and shake for 30 minutes.~~

**Weigh 0.500 g of ground tobacco directly into a disposable specimen bottle. Add 100 mL of 5% acetic acid extraction solution. Cap and shake at 200 rpms on the gyratory shaker for 30 minutes.**

~~**7.3** Filter the extract through a Whatman N° 40 (or equivalent) filter paper, reject the first few $cm^3$ of the filtrate, then collect the filtrate in an analyzer cup.~~

**Pour extract into a glass disposable test tube. Push a Technicon Seraclear® filter into the test tube and analyze the filtrate.**



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**7.4** Run the samples and standards through the system in the normal manner (*e.g.* priming with 6 4 tobacco extracts, calibration standards and samples ~~with 1 intermediate calibration solution after every 6 samples~~). If sample concentrations lie outside the range of the standards, the samples shall be diluted and run again.

## 8. Calculation

**8.1** Plot a graph of peak height against equivalent nicotine concentrations for all the calibration solutions.

**8.2** ~~Calculate the percentage nicotine (dry weight basis) in the tobacco using the formula:~~

$$\text{~~\% Nicotine (dwb)~~} = \frac{\text{~~c x V x 100~~}}{\text{m}} \times \frac{\text{~~100~~}}{\text{~~100-M~~}}$$

~~c is the nicotine concentration, expressed in milligrams per millilitre, obtained from the calibration curve (8.1);~~
~~V is the volume, in millilitres, of extract prepared (7.2) (normally 25 millilitres);~~
~~m is the mass, in milligrams, of the sample (7.2);~~
~~M is the moisture content, expressed as percentage by mass, of the tobacco (7.1).~~

**Weight % Alkaloids = mg nicotine/mL x 100 mL x 100%**
**mg sample**

**"Weight % Alkaloids is expressed as g of nicotine per 100 g of filler"**

**Nicotine (mg/g) = g nicotine x 1000 mg**
**100 g filler g**

The test result shall be expressed to two decimal places.

**Notes :**

**3** When using 5% acetic acid extracts the standard nicotine solutions (5.9) must be made up with 5% acetic acid and the wash cycle must be with 5% acetic acid.



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4 If this method is performed simultaneously with CORESTA Recommended Method N° 36, CORESTA Recommended Method N° 37 or CORESTA Recommended Method N° 38 combined standards may be prepared.

## 9. Repeatability and Reproducibility

An international collaborative study involving 12 laboratories and 3 samples conducted in 1993 showed that when single grades of tobacco were analyzed by this method, the following values for repeatability (r) and reproducibility (R) were obtained.

The difference between two single results found on different extractions by one operator using the same apparatus within a short time interval (the time it takes to analyze 40 sample cups) and without recalibration of the equipment during the time of analysis will exceed the repeatability value (r) on average not more than once in 20 cases in the normal and correct operation of the method.

Single results reported by two laboratories will differ by more than the reproducibility value (R) on average not more than once in 20 cases in the normal and correct operation of the method.

Data analysis gave the estimates as summarized in table 1 and 2.

**Table 1 : Extraction with water**

| Tobacco Type | Mean Content of Nicotine %(dwb) | Repeatability Conditions r | Reproducibility Conditions R |
|---|---|---|---|
| Oriental | 1.17 | 0.05 | 0.19 |
| Flue-Cured | 2.90 | 0.08 | 0.41 |
| Burley | 3.97 | 0.12 | 0.55 |

**Table 2 : Extraction with 5% Acetic Acid**

| Tobacco Type | Mean Content of Nicotine %(dwb) | Repeatability Conditions r | Reproducibility Conditions R |
|---|---|---|---|
| Oriental | 1.17 | 0.07 | 0.21 |
| Flue-Cured | 2.90 | 0.11 | 0.67 |
| Burley | 3.97 | 0.13 | 0.97 |

For the purpose of calculating r and R, one test result was defined as the yield obtained from analyzing a single extract once.



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## Appendix 1

Several collaborative studies, carried out by a CORESTA Task Force during 1989 and 1990, have shown that two other procedures give equivalent results to the Recommended Method. It may be necessary to use one of these alternative procedures, if so, the following comments should be considered before use:

- Cyanogen chloride can be alternatively generated *in situ* by the reaction of potassium thiocyanate and sodium hypochlorite. In order for this reaction to be successful the sodium hypochlorite must have an available chlorine content of 10% - 14% (m/m). It has been found that sodium hypochlorite with this amount of available chlorine is sometimes difficult to obtain.

- Cyanogen bromide in reaction with aniline can also be used in the determination of total alkaloids. Because of the hazardous nature of the cyanogen bromide, some countries have found problems with the importation and use of this substance.

## Appendix 2

### Preparation of a Microbore ~~Five Turn~~ Mixing Coil

~~1. Loop a standard orange white (0.64 mm id) pump tube 5 times around a glass tube (e.g. test tube, glass rod) with an external diameter of approximately 12 mm.~~

~~2. While holding the loops in place brush them thoroughly with cyclohexanone.~~

~~3. Use adhesive tape to hold the loops in place while the cyclohexanone sets the tubing, (about 10 hours).~~

~~4. Remove the glass tube from the coil.~~

**1. Coil 20 inches of 0.040" ID EVA (ethylvinyl acetate) tubing.**

~~**Diagrams**~~

~~**Diagram 1**~~
~~See picture in file RM35_01.PCX, repertory IMAGES.~~



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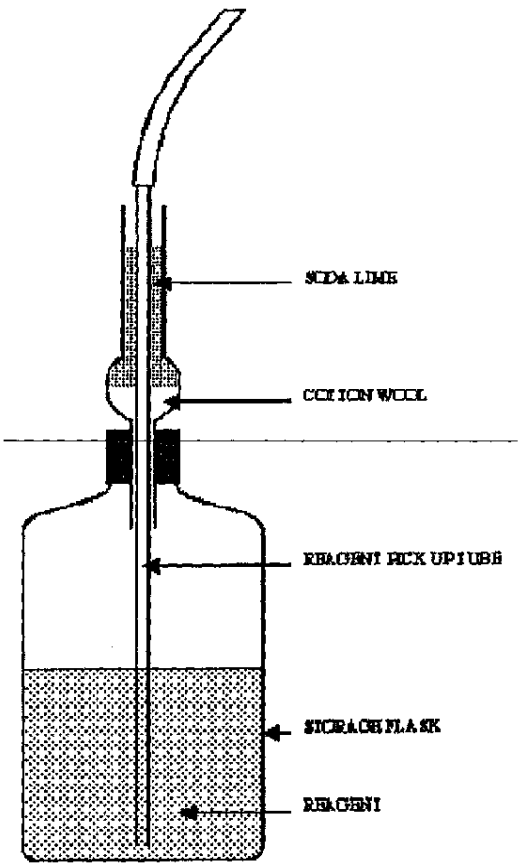
~~**Diagram 2**~~
~~Soda Lime Trap~~



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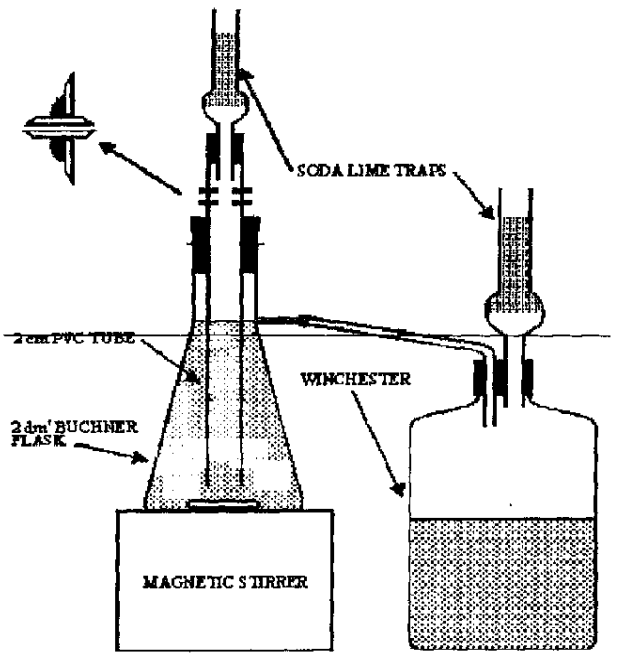
~~**Diagram 3**~~
~~On-Line Cyanogen Chloride Destruction Apparatus~~




~~The apparatus consists of a 2 $dm^3$ Buchner flask on a magnetic stirrer, with a 2 cm diameter PVC tube inserted into it, through a rubber bung, such that the tube is just above the magnetic follower in the flask. Four holes are drilled in the tube and nipples attached by gluing into position. The pullback line and the debubble line containing the cyanogen chloride are attached to the nipples, together with the two neutralising agents. This arrangement ensures that the cyanogen chloride has to pass down the tube and through the bulk of the flask before overflowing to waste, thus ensuring complete neutralisation.~~



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